

Characterization of bovine FAS-associated death domain gene¹

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Summary

The FAS-associated death domain (FADD) protein is an adapter/signaling molecule that has been shown to function in human cells to promote apoptosis and to inhibit NF- κ B activation. Because of the critical role that apoptosis and NF- κ B play in a variety of disease states, we mapped the bovine *FADD* gene, sequenced bovine *FADD* cDNA, and characterized its expression in endothelial cells (EC). Sequencing of bovine *FADD* revealed approximately 65 and 58% amino acid sequence identity to its human and murine homologues, respectively. Bovine *FADD* was mapped to chromosome 29 by radiation hybrid mapping. In addition, the functionality of bovine FADD was studied. Expression of a bovine FADD dominant-negative construct blocked bacterial lipopolysaccharide (LPS)- and TNF- α -induced apoptosis in bovine EC consistent with previous studies of human FADD. In contrast to human FADD, elevated expression of bovine FADD had no effect on LPS- or TNF- α -induced upregulation of NF- κ B-dependent gene products as assayed by E-selectin expression. Thus, while the role of FADD in mediating apoptosis is conserved across species, its role in regulating NF- κ B-dependent gene expression is not.

Keywords apoptosis, caspase, cytokines, endotoxin, inflammation.

Inflammation and apoptosis contribute to the pathogenesis and/or deleterious outcomes associated with many bacterial-mediated diseases including bovine mastitis (Capuco *et al.* 1986; Bayles *et al.* 1998; Bannerman *et al.* 2004). In humans and mice, the FAS-associated death domain (FADD) protein has been demonstrated to function as an adapter protein that couples receptors, such as those for bacterial lipopolysaccharide (LPS) and the pro-inflammatory cytokine TNF- α , to a family of proteolytic enzymes referred to as caspases (Chinnaiyan *et al.* 1996; Choi *et al.* 1998; Yeh *et al.* 1998). FADD recruitment to death receptor signaling complexes is mediated through highly conserved death domains (DD) contained within FADD and either the receptors, themselves, or DD-containing intermediaries recruited to the receptors (Chinnaiyan *et al.* 1995; Hofmann & Tschopp 1995). FADD, in turn, can recruit pro-caspase-8 via protein–protein interactions of death effector domains (DED) contained within both proteins (Muzio *et al.* 1996, 1998). FADD recruitment of pro-caspase-8 molecules

enables activation of these pro-enzymes into fully functional proteases resulting in the onset of apoptosis (Muzio *et al.* 1998).

NF- κ B is a key pro-inflammatory molecule that promotes the expression of cytokines and adhesion molecules (Tak & Firestein 2001). In addition to its role in promoting apoptosis, human FADD has been established to downregulate LPS-, but not TNF- α -induced NF- κ B activation (Chinnaiyan *et al.* 1996; Hsu *et al.* 1996; Bannerman *et al.* 2002). Because of the important role of FADD in mediating pro-apoptotic and pro-inflammatory signaling, we decided to investigate whether bovine FADD, similar to its human and murine homologues, is able to function as a pro-apoptotic adapter protein and whether bovine FADD could moderate NF- κ B dependent gene expression.

The complete coding sequence of bovine *FADD* cDNA (GenBank accession no. AY725483) was obtained by reverse transcription polymerase chain reaction (RT-PCR) using total RNA isolated from cultured bovine aortic endothelial cells (EC) (generous gift of Dr L.M. Sordillo, Michigan State University, East Lansing, MI, USA) as template. *FADD*-specific primers for RT-PCR (5'-CTGGTA GCAAAACGGCATTC-3' and 5'-GAGCTCGTTTAATGGGG ACA-3') were designed from bovine expressed sequence tags (TC290893) available in the *Bos taurus* Gene Index (<http://www.tigr.org/tdb/tgi>). Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) and subsequent PCR was done using

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2 µl of first-strand cDNA and iQ Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. Cycling conditions were 95 °C for 3 min, followed by 30 cycles of: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resulting 887-bp product was cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced using M13 primers. All sequencing was performed on a CEQ8000 automated DNA sequencer (Beckman Coulter, Fullerton, CA, USA). The bovine *FADD* cDNA encodes a predicted 209 amino acid protein sharing 65 and 58% amino acid sequence identity with human and murine sequences, respectively.

For physical mapping of the bovine *FADD* gene, targeted PCR amplification of a 490-bp region of putative exon 2 (based on homology with human genomic sequence) was carried out using primers 5'-CTGTGATAACGTGGGGAA GG-3' (sense) and 5'-TGGAGGAGCTCGTTTAATGG-3' (antisense) to amplify DNA from 94 clone lines of a 3000-rad bovine radiation hybrid (RH) panel (ResGen; Invitrogen). Amplification conditions for PCR were as follows: 10 cycles of 94 °C for 30 s, 65 °C for 30 s (-1 °C/cycle), 68 °C for 2 min; then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min; with a final extension of 68 °C for 10 min. The reaction products were scored by agarose gel electrophoresis and concordancy data for each cell line were submitted to the Roslin RH database (<http://databases.roslin.ac.uk.radhyb>) for two-point analysis using a modification of the RHmap Program (Lange *et al.* 1995). Using this approach, *FADD* was mapped to BTA29 nearest marker BMS1948 at approximately 61.6 cM (LODS = 13.87, distance = 0.16 cR₃₀₀₀).

To overexpress a full-length or dominant-negative (D/N) construct of bovine *FADD*, PCR was used to generate products with compatible ends for insertion into the pCR3.V64 Met FLAG vector (generous gift of Dr Jurg Tschopp, Institute of Biochemistry of the University of Lausanne, Switzerland) to yield coding regions with an initiator methionine, FLAG tag (DYKDDDDK), and a spacer of two amino acids (EF) before the start of the desired coding region. Template for PCR was the cloned coding region of bovine *FADD*. The primers used to generate full-length *FADD* (AA 1–209) were 5' CCG GAA TTC ATG GAC CCG TTC CTG GTG CTG 3' and 5' G CTC TAG AGC GGC CGC CGC TGG GTC GTC AGG AGG CT 3'. The *FADD* D/N construct encoding just the death domain of bovine *FADD* (AA 91–209) was amplified using 5' CCG GAA TTC GCG CCG GAG GAC CGA GAC CTG 3' and 5' G CTC TAG AGC GGC CGC CGC TGG GTC GTC AGG AGG CT 3'. Cycling conditions were as follows: 94 °C for 1 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2 min, followed by 10 min at 68 °C. The PCR products were digested with *EcoRI* and *XbaI*, and ligated into the pCR3.V64 Met FLAG vector. Bacteria were transformed with the ligation reactions and colonies positive by restriction digest for correct size inserts were fully sequenced. Correct clones were then

subcloned into the *BamHI* and *NotI* restriction sites of the retroviral expression vector pBMN-IRES-PURO (kindly provided by Dr Gary Nolan, Stanford University, Stanford, CA and subsequently modified by Dr Kyle Garton, University of Washington, Seattle, WA, USA).

High-titre retrovirus was used to infect EC with viral particles containing the expression vectors as previously described (Bannerman *et al.* 2002). Twenty-four hours post-infection, growth media was supplemented with 2 µg/ml puromycin (Sigma Chemical Co., St Louis, MO, USA) to select for cells stably expressing the construct of interest. Flow cytometry demonstrated that ~99% of the cells infected with the control vector encoding enhanced green fluorescent protein (EGFP) were positive for EGFP expression following puromycin selection (data not shown). Western blotting performed as previously described (Bannerman *et al.* 2002) with 1:2000 diluted anti-FLAG antibody conjugated to horseradish peroxidase (Sigma Chemical Co., St Louis, MO, USA) confirmed expression of both the *FADD* D/N (Fig. 1a) and *FADD* full-length constructs (Fig. 2a).

To determine whether bovine *FADD* participates in pro-apoptotic signaling, a truncated construct of bovine *FADD*, encoding solely the DD and that corresponds to a human construct previously demonstrated to function in a D/N manner (Choi *et al.* 1998), was expressed in bovine EC. Cells expressing either the EGFP control vector or the *FADD* D/N were treated with 100 ng/ml of TNF- α or LPS for 8 or 12 h, respectively, and assayed for caspase activity as previously described (Erwert *et al.* 2002). In those experiments where TNF- α -induced apoptosis was assayed, cells were sensitized by co-incubation with 40 µg/ml of cycloheximide. All experiments were performed in triplicate. Control EC expressing EGFP were sensitive to LPS- or TNF- α -induced apoptosis as evidenced by increased caspase activation following treatment (Fig. 1b and c). In contrast, EC expressing the bovine *FADD* D/N construct were almost completely resistant to LPS and TNF- α -induced apoptosis. These data demonstrate a role for bovine *FADD* in mediating LPS and TNF- α pro-apoptotic signaling.

In addition to its role in promoting apoptosis, human *FADD* has been reported to downregulate NF- κ B, the latter of which regulates the expression of several pro-inflammatory gene products (Bannerman *et al.* 2002). Specifically, increased expression of full-length human *FADD* has been shown to inhibit NF- κ B-dependent gene expression. To determine whether bovine *FADD* could similarly influence NF- κ B, EC overexpressing full-length bovine *FADD* were exposed to 100 ng/ml of LPS or TNF- α for 8 h and assayed by ELISA for E-selectin, the expression of which is regulated by NF- κ B (Tak & Firestein 2001). The results from three independent experiments demonstrated that exposure to LPS or TNF- α resulted in increased expression of E-selectin that was comparable among control EC and those overexpressing full-length bovine *FADD* (Fig. 2b and c). Thus, in contrast to that reported for human *FADD*, increased

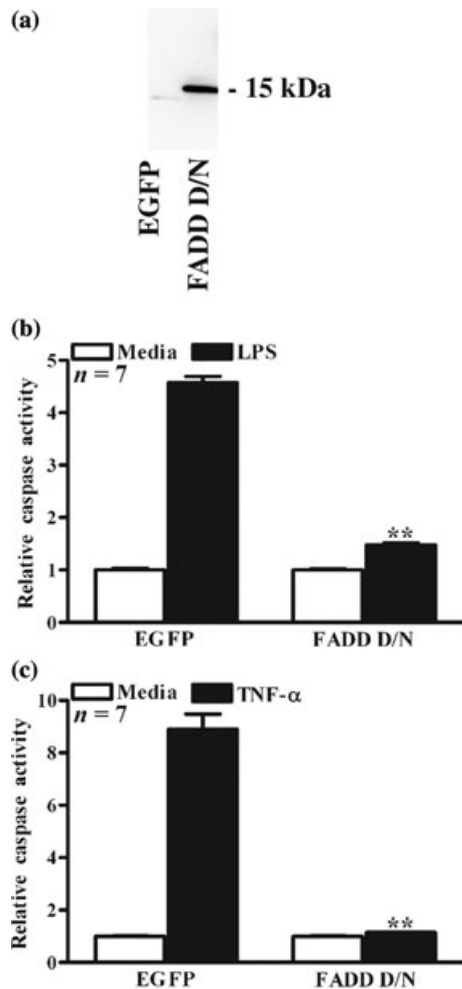


Figure 1. Effect of expression of a bovine FAS-associated death domain (*FADD*) dominant-negative construct on LPS- and TNF- α -induced apoptosis. Endothelial cells were stably transfected with either a control vector encoding enhanced green fluorescent protein (EGFP) or cDNA encoding the death domain of bovine *FADD* (a, b, c). An anti-FLAG antibody was used to confirm expression of this dominant-negative (D/N) *FADD* construct (a). In other experiments, cells were treated with media, LPS (100 ng/ml; 12 h) (b), or TNF- α (100 ng/ml; 8 h) (c), and assayed for caspase activity. Vertical bars represent mean (\pm SE) caspase activity relative to simultaneous media controls. **Significantly decreased compared with EGFP-vector transfected EC exposed to identical treatment.

expression of bovine *FADD* has no discernible effect on NF- κ B-dependent gene expression.

In conclusion, the bovine *FADD* cDNA was sequenced, mapped, and the protein functionally characterized in EC. The data presented here suggest that bovine *FADD* functions as a pro-apoptotic signaling molecule that lacks a dual role in moderating NF- κ B-dependent gene expression. This lack of an inhibitory role for bovine *FADD* on the pro-inflammatory NF- κ B pathway may explain the exquisite sensitivity of cattle to LPS-induced inflammation relative to other species (Berczi *et al.* 1966). Further studies will be required to investigate this hypothesis, as well as to evaluate

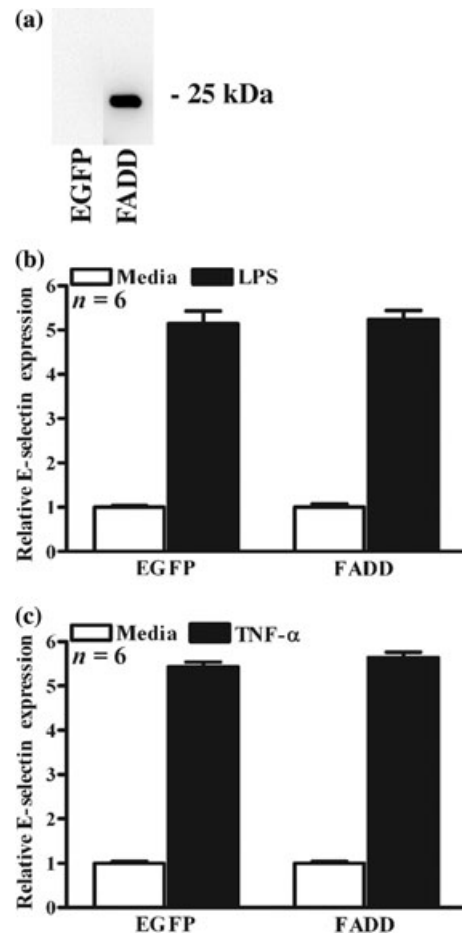


Figure 2. Effect of overexpression of bovine FAS-associated death domain (*FADD*) on LPS- and TNF- α -induced expression of the NF- κ B-regulated gene product, E-selectin. Bovine endothelial cells were stably transfected with either EGFP-vector alone or full-length bovine *FADD* (a, b, c), and expression confirmed by Western blot analysis using anti-FLAG antibodies (a). In separate studies, cells were treated for 8 h with media, LPS (100 ng/ml) (b), or TNF- α (10 ng/ml) (c), and assayed by ELISA for E-selectin expression. Vertical bars represent mean (\pm SE) E-selectin expression relative to simultaneous media-alone exposed cells.

the role of *FADD* in mediating apoptosis in disease states, such as Gram-negative mastitis, where the underlying pathogenesis is mediated by LPS and the pro-inflammatory cytokine TNF- α .

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